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Nicol, Samantha; Sabbah, Shereen; Jung, Jae; Brulios, Kevin; Bell, Andrew; Hislop, Andrew

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1 Primary B lymphocytes infected with KSHV can be expanded in vitro and are recognized by
2 LANA-specific CD4+ T cells.

3 Samantha M. Nicol,^a Shereen Sabbah,^b Kevin F. Brulois,^c Jae U. Jung,^c Andrew I. Bell,^d and
4 Andrew D. Hislop^{a#}

5 Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, UK^a;
6 Department of Immunobiology, King's College London, London, UK^b Department of
7 Molecular Microbiology and Immunology, Keck School of Medicine, University of Southern
8 California, Los Angeles, California, USA^c; Institute of Cancer and Genomic Sciences,
9 University of Birmingham, Birmingham, UK^d

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13 # Address correspondence to Andrew D Hislop, a.d.hislop@bham.ac.uk

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18 **Abstract**

19 Kaposi sarcoma-associated herpesvirus (KSHV) has a tropism for B lymphocytes in which it
20 establishes latency and can also cause lymphoproliferative disorders of these cells
21 manifesting as primary effusion lymphoma (PEL) and multi-centric Castleman disease
22 (MCD). T cell immunity is vital for the control of KSHV infection and disease however few
23 models of B lymphocyte infection exist to study immune recognition of such cells. Here we
24 developed a model of B lymphocyte infection with KSHV where infected tonsillar B
25 lymphocytes were expanded by providing mitogenic stimuli and these challenged with
26 KSHV-specific CD4⁺ T cells. Infected cells expressed viral proteins found in PELs namely
27 LANA and vIRF3, albeit at lower levels, with similar patterns of gene expression for the
28 major latency, vIL-6 and vIRF3 transcripts. Despite low level expression of ORF50,
29 transcripts for the immune evasion genes K3 and K5 were detected, with some
30 downregulation of cell surface expressed CD86 and ICAM. The vast majority of infected
31 lymphocytes expressed IgM and the Ig λ light chain, recapitulating these features seen in
32 infected cells in MCD. We assessed the ability of the infected lymphocytes to be targeted by
33 a panel of MHC class II matched CD4⁺ T cells and found that LANA-specific T cells
34 restricted to different epitopes recognized these infected cells. Given that at least some
35 KSHV latent antigens are thought to be poor targets for CD8⁺ T cells, we suggest that CD4⁺
36 T cells are potentially important effectors for the in vivo control of KSHV infected B
37 lymphocytes.

38

39 **Importance**

40 KSHV establishes a latent reservoir within B lymphocytes but few models exist to study
41 KSHV-infected B cells other than the transformed PEL cell lines which have likely accrued

42 mutations during the transformation process. We developed a model of KSHV-infected
43 primary B lymphocytes which recapitulate features seen in PEL and MCD by gene
44 expression and cell phenotype analysis, allowing the study of T cell recognition of these cells.
45 Challenge of KSHV-infected B cells with CD4+ T cells specific for LANA, a protein
46 expressed in all KSHV-infected cells and malignancies in vivo, showed that these effectors
47 could efficiently recognize such targets. Given the virus expresses immune evasion genes or
48 use proteins with intrinsic properties such as LANA that minimize epitope recognition by
49 CD8+ T cells, CD4+ T cell immunity to KSHV may be important for maintaining the virus
50 host balance.

51

52 **Introduction**

53 Kaposi sarcoma-associated herpesvirus (KSHV) is one of the two human γ -herpesviruses
54 with oncogenic potential. This virus has a tropism for endothelial cells where it is associated
55 with the development of Kaposi sarcoma (KS), as well as being tropic for B lymphocytes
56 where it can cause primary effusion lymphoma (PEL) and multi-centric Castleman disease
57 (MCD) (1). The immune response is important for control of KSHV infection as infected
58 patients whose cellular immune response is suppressed either for transplantation or due to
59 untreated HIV infection are at an increased risk of developing KS. Importantly in these
60 scenarios if immune competence is restored through relaxation of immunosuppression or
61 administration of highly active anti-retroviral therapy respectively, regression of KS lesions
62 can be seen, implying an important role for the T cell response in control of the virus and
63 malignancies (2, 3).

64 KSHV is known to infect CD19⁺ B lymphocytes (4), but little is known how KSHV-specific
65 T cell control is exercised over infected B lymphocytes. The only model which has been
66 used to examine T cell recognition of infected B lymphocytes comes from studies using PEL
67 derived lines as targets. Here CD8⁺ T lymphocytes were unable to recognize reporter
68 antigens expressed in PELs thought to be related to their low expression of the transporter
69 associated with antigen processing-1 mRNA, disrupting antigen presentation to these
70 effectors (5). CD4⁺ T cells specific to the genome maintenance protein LANA, a protein
71 expressed in all infected cells and malignancies, in most cases showed poor if any recognition
72 of PELs (6). This was a consequence of expression of the KSHV gene vIRF3 which,
73 amongst other functions, inhibits expression of the MHC class II transcriptional transactivator
74 CIITA; a protein required for the expression of class II and other genes in this antigen
75 processing pathway (7). Although these studies are performed on cell lines derived from

76 patients with disease, these lines have likely accrued mutations and may not resemble B cells
77 which the virus maintains latency in. The ability of KSHV-specific T lymphocytes to respond
78 to KSHV infected non-transformed B lymphocytes which are likely to have intact antigen
79 processing pathways is so far largely untested.

80 Several models of primary B cell infection have been developed to study KSHV infection in
81 these cells. Initial studies used CD40 ligand stimulation of B cells to make them receptive to
82 infection (8), while others have co-cultured B lymphocytes with virus producer cells to allow
83 direct cell to cell virus transfer, or exposed B lymphocytes to concentrated preparations of
84 KSHV (9-12). Such in vitro infected cells express latent transcripts and transiently express
85 selected lytic transcripts and genes, inducing some proliferation of the infected cells but
86 unlike the related γ -herpesvirus Epstein-Barr virus (EBV), does not lead to transformation
87 (10). These models have been informative for studying which cytokines may be induced by
88 infection (13) and examining the potential identity of B lymphocyte targets of KSHV, which
89 are likely IgM⁺ CD27⁺ Ig λ using cells (10).

90 However the analysis of immune recognition of ex vivo infected B cells has been limited
91 with studies suggesting that CD4⁺ T cells may suppress spontaneous lytic replication and
92 encourage latency (11). This mechanism required cell contact, the CD4⁺ T cells to be
93 activated, but was independent of MHC restriction and so how such restriction might operate
94 in vivo is not clear. Furthermore, how ex vivo infected cells which have stably entered
95 latency may be controlled by KSHV-specific immune effectors has not been tested. To
96 examine these questions we developed a KSHV B cell infection model where we expanded
97 infected cells to ask what viral genes are expressed in infected cells, how infection may
98 modulate immune receptor expression and whether antigen-specific T cells can recognize
99 these targets.

100

101 **Materials and Methods**102 Tonsil cell preparations and infections

103 Tonsil specimens were obtained from patients undergoing routine tonsillectomy to treat
104 chronic tonsillitis. Patients were adolescent or young adults and their tonsils were not
105 inflamed at the time of surgery. All participants gave written informed consent in accordance
106 with the Declaration of Helsinki; ethical approval was granted from the South Birmingham
107 Health Authority Local Research Ethics Committee. Specimens were disaggregated to single
108 cell suspensions by teasing apart the tissue and fine mincing. Mononuclear cells were
109 isolated by purification over a Lymphoprep gradient (Nycomed Pharma), as per the
110 manufacturer's instructions, aliquoted, cryopreserved and stored in liquid nitrogen. DNA
111 was isolated from an aliquot of the tonsillar cells for HLA typing by sequence specific
112 oligonucleotide PCR analysis performed at the Anthony Nolan Trust.

113 Tonsillar mononuclear cells were infected with KSHV using a protocol similar to one
114 previously described (14). Briefly rKSHV.219 infected Vero cells (VK219; (15)) transduced
115 with a pInducer 20 lentivirus (16) engineered to express ORF50 under the control of the
116 tetracycline promoter, or ORF50 lentivirus transduced Vero cells infected with either the
117 BAC16 derived K5 deletion mutant or its paired revertant virus (17), were seeded in 24 well
118 plates at 50 000 cells per well. After 24 hours, virus replication was induced for 24 hours by
119 the addition of 2 µg/ml doxycycline and 1.25 mM sodium butyrate. Media from these wells
120 was then removed and 500 000 tonsillar mononuclear cells were seeded per well, centrifuged
121 onto the VK219 monolayers and incubated for 48 hours. Parallel mock infections of tonsillar
122 cells were conducted by culturing these either on monolayers of VK219 cells which had been
123 treated with 1 mM phosphonoacetic acid (18) for the previous 30 hours prior to induction and

124 during the co-culture, or culturing the tonsillar cells in the absence of VK219 cells. B cells
125 were then purified from the mock and KSHV infected cultures using anti-CD19 Dynabeads
126 (LifeTechnologies) and the beads removed using a Detachabead CD19 kit as per the
127 manufacturer's instructions.

128 Purified B cells were cultured in Iscoves MEM with 100 U/ml IL-4 (Peprotech), penicillin
129 streptomycin, gentamycin and Fungizone (Life Technologies) on monolayers of L cells
130 transduced to express CD40L that had been γ -irradiated (10 000 rads). After 48 hours
131 KSHV-infected cells were selected by addition of puromycin to a final concentration of
132 between 0.1-0.3 μ g/ml. B cells were expanded, moved to fresh L cell monolayers weekly
133 and maintained under puromycin selection.

134 Presence of contaminating VK219 cells in the B lymphocyte cultures was determined by
135 performing semi-quantitative end point PCR assays on 25 ng of DNA extracted from the
136 cultures. The PCR assay detected the neomycin resistance gene encoded by the ORF50
137 lentivirus used to transduce the VK219 cells. Primers used were neo forward AGG ATC TCC
138 TGT CAT CTC ACC TTG CTC CTG and neo reverse AAG AAC TCG TCA AGA AGG
139 CGA TAG AAG GCG. As controls, transduced VK219 cells were mixed with BJAB cells to
140 give 0.1% and 0.01% VK219 cells and DNA extracted from these mixtures. These controls
141 and DNA from the samples was subject to PCR amplification using different numbers of
142 cycles to detect products.

143 B cell surface marker expression was assessed by staining cells with antibodies specific to
144 HLA class I, HLA-DR, CD20, CD54, CD86 or appropriate isotype control antibodies
145 (Biolegend). Ig λ and Ig κ expression was assessed by staining cells with biotinylated
146 antibodies specific to these proteins (Southern Biotech) and these detected by incubating with
147 avidin conjugated APC-Cy7 (Biolegend). Cells were fixed in 2% paraformaldehyde

148 (eBioscience), analyzed on an LSRII flow cytometer (Becton Dickinson) and data processed
149 using Flowjo (Treestar).

150 Immunofluorescence staining for LANA protein expression in KSHV infected B cells

151 Established KSHV infected or mock infected B cell lines were assayed for LANA protein
152 expression by immunofluorescence staining. Cell suspensions were washed in phosphate
153 buffered saline (PBS), dried onto microscope slides and fixed in cold acetone for 10 minutes.
154 Slides were then dried, washed in PBS and stained with either an isotype control rat antibody
155 or a LANA-specific rat antibody (clone LN 53, Advanced Biotechnologies) for 1 hour at
156 37°C. Slides were washed four times and bound antibody detected using an anti-rat Alexa
157 568 conjugated antibody (Life Technologies) and incubating for 1 hour at 37°C. Slides were
158 then washed and examined using a Nikon E600 microscope fitted with epifluorescence
159 detection.

160 Western blot analysis

161 Cells were lysed in 9M urea with 0.075 M Tris HCl, pH 7.5, sonicated and lysates clarified
162 by centrifugation. Protein concentrations were determined by Bradford assay (Bio-Rad
163 Laboratories) and 20 µg of protein separated by SDS-PAGE and transferred onto
164 nitrocellulose membranes using standard techniques. Blots were probed with antibodies
165 specific for LANA (clone LN53), vIRF3 (CM-A807, Abcam) and actin (AC-74, Sigma-
166 Aldrich). Bound antibodies were detected using the appropriate anti-species peroxidase
167 labelled antibody, followed by detection using an ECL kit (GE Healthcare).

168 Measurement of viral transcripts

169 Total RNA was extracted from cells using a NucleoSpin RNA II kit (Macherey Nagel)
170 according to the manufacturer's instructions. An aliquot (1 µg) was treated with DNase I

171 (Life Technologies) to remove residual genomic DNA, before being reverse-transcribed
172 using Qscript (VWR).

173 Selected KSHV transcripts were quantified by Taqman qRT-PCR using the primer and probe
174 combinations shown in Table 1. Primer and probe sequences were designed using Primer
175 Express 3.0 (Life Technologies) and were based on the BC-1 KSHV genome sequence
176 (accession number U75698). Primer-probe combinations were selected to avoid known
177 KSHV sequence polymorphisms and assays to detect spliced transcripts were designed to
178 span exon-exon junctions. All Taqman probes (Eurogentec) were modified with FAM and
179 TAMRA at the 5' and 3' ends, respectively. Cellular GAPDH mRNA, used as an internal
180 control, was detected using a VIC-labelled commercial assay (Life Technologies, 4310884E).

181 Amplification reactions were prepared in a final volume of 25 µl containing 1 x Taqman
182 Universal MasterMix II (Life Technologies), 300 nM forward and reverse primers, 200 nM
183 probe, 0.5 µl GAPDH reagent and 5 µl cDNA. PCR amplifications were performed using an
184 ABI 7500 with default thermocycling conditions. All test samples were run in duplicate,
185 while template-negative and RT-negative samples served as controls. To determine the
186 absolute levels of KSHV and GAPDH transcripts, serial dilutions from 1 – 10⁵ copies of a
187 plasmid (AQ2) were included in each PCR experiment and used to generate appropriate
188 standard curves. AQ2 was derived from the AQ plasmid (19) by the insertion of a
189 commercially synthesised 1093 bp sequence carrying the contiguous KSHV amplicons
190 (GenScript). All data were analysed using Sequence Detection Software v2.0 (Applied
191 Biosystems) and are reported as copies relative to GAPDH.

192 Comparisons of gene expression levels between PELs and infected B lymphocytes were
193 performed using the R statistical program (v 3.0.2) (20) on log₁₀ transformed mean values so
194 these were normally distributed as judged using the Shapiro-Wilk test. These transformed

195 values were then subjected to two sample t-tests to determine differences in transcript levels
196 between PEL lines and infected lymphocytes.

197 KSHV genome loads

198 DNA was extracted from cells using a NucleoSpin Tissue kit (Macherey Nagel) and viral
199 genome loads determined by qPCR. KSHV DNA was detected using the vIL-6 primer-probe
200 combination while cellular beta 2 microglobulin (B2m), used as an internal control, was
201 detected using published primers (21). Serial dilutions of AQ2 plasmid and BJAB DNA were
202 used to generate standard curves for vIL6 and B2m, respectively. Data are expressed as
203 KSHV genome copies per cell, assuming two B2m genes per diploid cell.

204 T cells and recognition experiments

205 The ability of T cells to recognize KSHV infected targets was performed as described
206 previously, using established T cell clones (6). Briefly, triplicate cultures of 5000 T cells
207 were incubated with 50 000 target cells which were either KSHV-infected or mock infected
208 target B cells, or B cells sensitized with the T cells cognate synthetic peptide-epitope
209 (Mimotopes). Cells were incubated in RPMI-1640 10% FCS for 18 hours and supernatants
210 then harvested from these cultures and assayed for IFN- γ by ELISA (Endogen).

211

212 **Results**

213 KSHV infection of primary B cells and their propagation.

214 In a preliminary set of experiments we determined whether we could infect tonsillar derived
215 B cells with rKSHV.219 virus. Unfractionated tonsillar mononuclear cells were infected with
216 KSHV by incubating on monolayers of Vero cells which contained latent rKSHV.219 that
217 had been treated 24 hours previously to induce virus replication. As a mock infection,
218 parallel aliquots of tonsillar cells were incubated on monolayers of induced VK219 cells
219 which had been treated for the previous 30 hours with phosphonoacetic acid to inhibit virus
220 production. After 48 hours co-culture, CD19 expressing B cells were selected, cultured for
221 72 hours to allow GFP expression from the rKSHV.219 genome, and the proportion of
222 infected cells identified by flow cytometry. Figure 1 shows two representative results of such
223 infections from tonsillectomy patients T46 and T7. Consistent with previous reports (9) we
224 found that these cells could be infected at a low percentage; typically GFP expressing cells
225 would be detected in the range of 0.5-1.6% of B cells.

226 We next asked whether the infected cells could be expanded by in vitro culture. Previous
227 studies had shown that unlike the related γ -herpesvirus Epstein-Barr virus (EBV), ex vivo
228 infection of B cells with KSHV does not lead to transformation of the B cells (9) but induces
229 some limited proliferation (10). To expand the population of B cells, we delivered a
230 mitogenic stimulus to them by incubating these on CD40-ligand expressing L cells in the
231 presence of IL-4. As rKSHV.219 virus encodes a puromycin resistance gene, we enriched
232 for infected B cells by selection with puromycin. We found that the infected cells were very
233 sensitive to puromycin requiring low doses to enrich these cells which did not obviously
234 affect L cell viability. However compared to mock infected B cells cultured in parallel
235 without puromycin, the infected cells grew slowly, doubling every 4-5 days as compared to

236 the parallel uninfected B cells which doubled every 72 hours. All subsequent experiments
237 used cells which had undergone at least three to four weeks of selection, which contained
238 between 5% to 50% GFP expressing cells. The presence of contaminating VK219 cells was
239 examined using a semi-quantitative PCR assay to detect the neomycin resistance gene
240 encoded by the ORF50 expressing lentivirus used to transduce these cells. Only DNA
241 extracted from lines derived from two of nine lines, T44b and T48, had detectable sequence
242 at levels less than 0.01% of the population (data not shown). B cell lines could be maintained
243 for a similar length of time compared to mock infected cells, typically for up to 12 weeks,
244 with some lasting >20 weeks. During passaging of these cells we monitored for RFP
245 expression, a gene in rKSHV.219 expressed under the control of the PAN promoter, as a
246 marker of lytic cycle replication and observed few cells, usually less than 5%, expressing this
247 marker in the cultures. To confirm that the B cells were infected with rKSHV.219, we
248 purified these by FACS for GFP expression and conducted immunofluorescence assays on
249 these cells staining for LANA. Figure 2 shows representative results from one of three sorts,
250 showing the characteristic punctate staining of LANA, reminiscent of the pattern seen in
251 primary effusion lymphomas (22), indicating these KSHV infected cells could be expanded
252 in vitro.

253

254 Antigen and gene expression of KSHV infected primary B cells

255 In the next series of experiments we sought to determine which KSHV genes were expressed
256 in the infected B cells, principally to identify which could be used as immunological targets
257 and also monitor any immune evasion gene expression. We focused on likely latent genes
258 and their products as we observed few RFP expressing cells in our established cultures,
259 suggesting no substantial spontaneous lytic reactivation was occurring. Western blot analysis

260 was performed on lysates from two tonsillar preparations of rKSHV.219 infected B cells
261 which had been FACS sorted to 90% purity or mock infected B cells maintained on CD40-
262 ligand and IL-4 in parallel. As a control, a lysate from the PEL JSC-1, from which
263 rKSHV.219 was derived was used. Blots were initially probed with antibodies to vCyclin
264 and vFLIP however no expression of these proteins was detected in the JSC-1 or any B cell
265 lysates, nor could we detect expression of the EBV protein EBNA1, found in approximately
266 80% of PEL lines, in infected B lymphocyte lysates (data not shown). Blots were then
267 sequentially probed for LANA, vIRF3 and actin, the latter as a loading control, and results
268 shown in Figure 3. Infected B cells demonstrated expression of LANA, although at levels
269 lower than seen in JSC-1, while vIRF3 expression in the infected B cells was substantially
270 lower than that in the PELs.

271 To further characterize these infected B cells we measured viral transcript expression by
272 qRT-PCR using a panel of Taqman based assays we developed. These included the splice
273 variants of transcripts from the major latency locus driven from the constitutive promoter as
274 shown in Figure 4A (23, 24), namely the tricistronic mRNA encoding LANA, vCyclin and
275 vFLIP, the bicistronic mRNA encoding vCyclin and vFLIP and the monocistronic mRNA
276 encoding vFLIP. Specificity of assays which detect spliced transcripts was confirmed by
277 testing against unspliced (genomic) DNA which showed no detection using the bicistronic
278 vCyclin vFLIP assay and weak detection using the monocistronic vFLIP assay, giving a
279 background of 0.13% compared to the unspliced tricistronic message. Assays measuring the
280 PEL expressed vIRF3 and vIL-6 mRNAs were also developed as were assays for three lytic
281 cycle expressed genes including the immediate early expressed lytic switch gene ORF50
282 (RTA) and two early expressed mRNAs K3 and K5. The location of the amplicons within
283 the genome, primer and probe sequences are shown in Table 1. To validate these assays we
284 tested them on RNA extracted from the PELs BC-1, JSC-1, BCBL-1, VG-1, and BC-3. All

285 assays were compared to a standard which was a synthetic plasmid containing the PCR
286 amplicons, thereby allowing absolute quantitation of transcript levels within a cell type.

287 Figure 4B (left panels) shows results of qRT-PCR assays reporting results relative to GAPDH
288 expression, grouping transcripts into either those expressed from the latency locus (LANA,
289 vCyclin, vFLIP), PEL specific transcripts (vIRF3 and vIL-6) and lytic transcripts (ORF50,
290 K3 and K5). Latency locus transcripts showed in most cases that the tricistronic transcript
291 was the most abundant followed by the bicistronic transcript, the exception being BC-1 where
292 this pattern was reversed. Previous northern blot analysis of BC-1 has showed a consistent
293 profile as to what we detected, but some contrast for BCBL-1, where northern analysis
294 indicated the bicistronic transcript to be more abundant than the tricistronic message (23, 25).
295 What dictates splicing of these RNAs is unknown and so why this difference has occurred is
296 not clear, but may relate to drift in gene expression patterns in cultured cells over time or be
297 related to the culture conditions used in our experiments. Interestingly the BCP-1 PEL, not
298 analyzed in our experiments, by northern analysis showed a similar pattern of transcript
299 expression as to what we have detected in most PELs with the tricistronic more abundant
300 than the bicistronic message (26). Low level expression of the monocistronic transcript was
301 detected, consistent with previous findings (24). All PELs expressed vIL-6 RNA, usually at
302 high levels compared to other transcripts, and all expressed vIRF3. In most cases low levels
303 of the ORF50 transcript were detected but perhaps surprisingly, transcripts for the early
304 expressed K3 and K5 genes were detected.

305 We next performed these qRT-PCR assays on RNA extracted from the infected B cells which
306 had been enriched for infection by selection with puromycin. These cells showed different
307 levels of KSHV infection as judged by GFP expression, thus: T3 90%, T10 90%, T13 75%,
308 T14 43%, T32 51%, T42 62%, T44a 51%, T44b 54% and T48 29%. To account for these
309 variations and allow comparison of transcript levels between the different infected cells and

310 PELs, we corrected transcript levels to 100% GFP expression. Figure 4B (right panels)
311 shows results of transcript levels in these cells where patterns of transcript expression showed
312 similarities to what was seen in the PELs. Thus the tricistronic was the most abundant of the
313 latency locus transcripts, followed by the bicistronic with very low levels of the
314 monocistronic transcripts detected. The vIL-6 transcripts were again the most abundant
315 detected, while there was lower and variable expression of vIRF3. Low levels of ORF50
316 transcript were detected, consistent with our observation of few if any RFP expressing cells
317 in the cultures. However similar to the PEL data, some expression of K3 and K5 transcripts
318 was detected in the infected B cells. Statistical analysis comparing individual transcript
319 levels between PELs and infected lymphocytes showed that only the tricistronic transcript
320 showed a significant difference between the two cell types (t-test, $p=0.023$). Overall, the
321 pattern of transcript level in the infected B cells broadly recapitulated what was detected in
322 the PELs.

323 PEL cell lines are known to harbor high frequencies of KSHV genomes so using our qPCR
324 assays we quantified genome load per cell in the infected B cells and compared these levels
325 to what we observed in PELs. Figure 4C shows results of these assays. PELs contained
326 variable but high levels of genomes with BC-3 containing over 600 copies of the genome,
327 while JSC-1, BCBL-1 and BC-1 had lower yet substantial frequencies of genomes, being
328 between 145 to 244 copies per cell. These values were comparable to those previously
329 reported using a similar methodology on these cell lines (27). Interestingly in repeated assays
330 on VG-1 DNA only two copies of the genome per cell were detected. Analysis of genome
331 loads of infected B cells, corrected for frequency of GFP expressing cells, showed these had
332 lower frequencies as compared to most PELs in five of seven lines analyzed, while lines
333 derived from donors T10 and T44a showed comparable or greater copies of genomes. A
334 second independently established line from this donor, T44b, showed levels closer to the

335 other infected cells. These findings suggest that that the infected B cells maintain similar or
336 reduced genome loads as compared to PELs.

337

338 Cell surface phenotype analysis of infected B cells

339 We next examined the B cells to identify phenotypic markers associated with infection and
340 whether there was evidence of altered immunological marker expression. Firstly the identity
341 surface immunoglobulin heavy and light chain expression was determined as in MCD, KSHV
342 is found in IgM positive λ light chain expressing cells (28). Here purified B cells which had
343 been exposed to the virus producer cells and then cultured for 72 hours to allow GFP
344 expression were stained for surface expression of Ig λ and IgM. Figure 5A shows flow
345 cytometry results of this analysis, where GFP negative uninfected cells showed there was
346 split expression of Ig λ and Ig κ (not shown) and that the majority of the population expressed
347 IgM. By contrast, few GFP positive cells showed Ig κ expression (data not shown), however
348 there was a marked if not exclusive preference for co-expression of Ig λ and IgM by the
349 infected cells. Figure 5B shows a similar analysis of established B cell lines which had been
350 maintained for more than 5 months. Mock infected cell lines contained both Ig λ and Ig κ (not
351 shown) expressing cells, however there were few IgM expressing cells in these cultures. The
352 KSHV infected cells however showed little evidence of Ig κ expression (not shown) but
353 expressed Ig λ and had maintained their expression of IgM, indicating that culturing these
354 cells in this fashion recapitulates phenotypic features seen in KSHV disease states.

355 KSHV encodes genes such as K3, K5 and vIRF3 which can modulate cell surface expression
356 of molecules which immune effectors recognize or receive co-stimulatory signals from (7,
357 29-32). As such we determined the cell surface expression of HLA class I, class II, and the

358 co-stimulatory molecules CD86 and CD54 (ICAM) on infected cells which had undergone
359 enrichment by puromycin selection and compared expression to co-resident uninfected cells
360 within the culture by flow cytometric analysis. Despite expression of K3 transcripts in
361 infected cells (Figure 4), no obvious modulation of this proteins target, namely surface HLA
362 class I, was observed (Figure 6A). Similarly infected cells showed little change in surface
363 levels of HLA class II as compared to uninfected cells, despite the presence of vIRF3
364 transcripts.

365 Analysis of T lymphocyte costimulatory marker expression is presented in Figure 6B where
366 compared to uninfected cells, infected cells had reduced surface levels of CD86 and CD54.
367 Such a profile is consistent with expression of the K5 gene as measured in the qRT-PCR
368 analysis, whose product has ubiquitin ligase activity which induces endocytosis of these
369 proteins. To test whether K5 was responsible for the downregulation of these markers, B
370 cells were infected with a recombinant KSHV in which the K5 gene was deleted (K5Δ) or a
371 derivative of this virus in which the K5 gene is restored (K5R) (17). Cells were selected and
372 surface marker analysis conducted as before; Figure 6C shows CD86 and CD54 surface
373 expression analysis results of one of three pairs of lines infected with the K5Δ or K5R
374 viruses. Cells infected with the K5Δ virus showed substantial but not complete restoration of
375 surface CD86 and CD54 expression as compared to co-resident uninfected cells, suggesting
376 that K5 expression alters at least some surface markers on infected cells in this model.

377

378 Recognition of KSHV infected B cells with KSHV-specific CD4⁺ T cells

379 As we had demonstrated that the KSHV-infected cells expressed LANA RNA and protein,
380 but did not appear to obviously modulate MHC class II cell surface expression, we
381 determined whether these infected cells could be targeted by a panel of LANA-specific CD4⁺

382 T cells. We were especially interested in the ability of CD4⁺ T cells to recognize this antigen
383 as it is expressed in all infected cells in vivo and the repeat sequences within LANA have
384 been shown to decrease presentation of epitopes to CD8⁺ T cells (33, 34). For these
385 experiments we HLA-typed the infected B cells and challenged these with HLA matched
386 CD4⁺ T cell clones specific for LANA which we had previously established (6). A total of
387 six T cell clones were used, three of which were responsive to the HLA-DQ6 presented
388 peptides LAPSTLRSLRKRRLLSPQGP (LAP/LRS), EYRYVLRTSPPHRPG (EYR) or
389 PAFVSSPTLPVAPIP (PAF), two clones which were specific for the HLA-DR13 presented
390 peptides LRSLRKRRLLSPQGP (LRS) or GDDLHLQPRRKHVAD (GDD), and one
391 responsive to the HLA-DQ7 presented peptide GSPTVFTSGLPAFVS (GSP). Here the
392 clones were incubated with either the infected B cells, mock infected B cells or mock infected
393 B cells sensitized with the T cells cognate peptide, for 18 hours and recognition assessed by
394 measuring IFN- γ secretion from the T cells. Figure 7 shows representative results from three
395 assays, where the effectors were challenged with B cell lines infected with KSHV or mock
396 infected lines that had been maintained on CD40L and IL-4 in parallel. T7 and T31 KSHV-
397 infected targets were 68% and 93% GFP positive respectively and these co-expressed both
398 HLA-DR13 and HLA-DQ6, while T10 infected targets were 23% GFP positive and
399 expressed HLA-DQ7. In all cases we observed IFN- γ secretion from the T cells when
400 challenged with the infected cells, indicating that despite the down regulation of cell surface
401 co-stimulatory molecules and the low level expression of vIRF3, these KSHV LANA-
402 specific CD4⁺ T cells could recognize the KSHV-infected B cells.

403

404 **Discussion**

405 In the present study we developed a model of primary B lymphocyte infection with KSHV to
406 examine gene expression and immune recognition of infected B cells which, unlike PEL cell
407 lines, have not gone through a transformation process. Infected B lymphocytes expanded
408 with mitogenic stimuli expressed latent proteins and genes in a similar pattern to what is
409 observed in PELs. Phenotypically these recapitulated features of MCD infected cells namely
410 the almost exclusive expression of IgM and Ig λ by the infected cells. Although these cells
411 expressed genes associated with immune evasion, some of which did modulate cell surface
412 markers, we found that the KSHV-infected cells were capable of being recognized by LANA-
413 specific CD4⁺ T cells.

414 Our initial studies characterizing the KSHV-infected B cells showed that they expressed key
415 proteins namely LANA and vIRF3, while qRT-PCR analysis indicated that a similar
416 repertoire of genes were expressed as seen in PELs assayed in parallel. However despite
417 finding transcripts for LANA and vIRF3 in the infected lymphocytes, the corresponding
418 levels of protein for each were lower than the PELs, suggesting that in this model, factors
419 other than transcript abundance affects protein levels. We have previously found that ectopic
420 expression of LANA or vIRF3 in model cell lines at levels similar to those in PELs is toxic
421 (unpublished observations). We speculate that PELs adapt to high level expression of these
422 proteins during the transformation process and that the infected lymphocytes in the present
423 study have not had sufficient time to adapt to high level protein expression. Some expression
424 of the lytic cycle assigned K3 and K5 genes was also observed, potentially related to the low
425 level expression of ORF50. However few cells expressed RFP, a marker of lytic cycle
426 replication, suggesting that either these transcripts were detected from the few cells with lytic
427 virus replication, or that these genes were being expressed outside of true lytic replication.

428 The latter case seems most likely as all infected lymphocytes, rather than a subset, showed
429 decreased surface expression of K5 target proteins. Furthermore, recent transcriptome
430 analysis of the PEL BCP-1 indicated that some transcripts, particularly K5, can be found in
431 these cells in the absence of obvious lytic cycle replication (35). Such expression patterns
432 show some contrast to what has been observed in latently infected SLK cells, where there is
433 minimal expression of K5 and modulation of its targets outside of lytic cycle replication (17).
434 K5 expression may then have been induced in our cells due to the culture conditions used, or
435 as PELs express this transcript, the K5 promoter may be activated in a B cell background, due
436 to B cell specific transcription factors. Alternatively this may be a consequence of the
437 elevated genome loads detected as high genome loads, at least in primary infection of cells,
438 are associated with the modulation of these cell surface immune cell stimulating ligands (36).

439 Despite the expression of K5 and the decreased surface expression of CD86 and CD54, no
440 obvious modulation of surface MHC class I was seen on the infected cells. This is likely due
441 to the apparent increased sensitivity of CD86 and CD54 to K5 as compared to MHC class I,
442 especially certain allotypes of class I (29, 31, 37). Turning to MHC class II expression, no
443 obvious decrease in cell surface expression of these proteins was observed on infected cells
444 despite the expression of vIRF3. This protein can block promoter activity of the class II
445 transcriptional transactivator CIITA, which is required for expression of MHC class II and
446 other genes required for the MHC class II processing pathway (7). Although vIRF3
447 transcript was expressed, we detected low levels of protein in infected cells compared to the
448 JSC-1 PEL. Interesting in this context we have previously found that BCBL-1 PELs
449 similarly express low levels of vIRF3 protein compared to other PELs, but maintain good
450 expression of surface MHC class II (6).

451 Analysis of cell surface marker expression showed that the vast majority of KSHV infected
452 cells were predominantly within the Ig λ light chain IgM heavy chain subset, reminiscent of

453 these features observed within infected cells of MCD lesions (28). Established cultures of
454 infected cells maintained this phenotype while parallel mock infected cultures switched
455 immunoglobulin heavy chain isotype usage, a likely consequence of continual CD40-ligand
456 and IL-4 stimulation, suggesting that virus infection suppresses isotype switching.
457 Interestingly this bias of light chain usage was seen within 72 hours after virus infection.
458 Related studies in which KSHV infection of tonsillar B lymphocytes was examined at 72
459 hours post exposure for LANA protein expression have also shown that the vast majority of
460 LANA expressing cells were in the Ig λ using population (10). Why this bias occurs is unclear
461 as there are no obvious functional or phenotypic differences between these two subsets.
462 Potentially this selection may be a consequence of vFLIP expression as B cells within a
463 transgenic vFLIP mouse model more frequently utilize this light chain (38). In this case the
464 ability of vFLIP to promote expression of NF- κ B may be responsible, as this transcription
465 factor is required at key stages of immature B cell development for the selection of Ig λ using
466 cells (reviewed in (39)). However as light chain rearrangement occurs during B lymphocyte
467 maturation in the bone marrow, the tonsillar B lymphocytes used in this study likely have
468 already undergone light chain selection. This may suggest that either infected cells may be
469 converted to Ig λ usage through processes such as receptor revision (40) although this may
470 seem unlikely given the vast majority of cells are expressing this receptor by 72 hours post
471 infection. Alternatively Ig κ using cells may not sustain infection.

472 The T lymphocyte response is vital for effective control of KSHV infection and disease,
473 however whether one subset of T lymphocytes, either CD4⁺ or CD8⁺, is more effective
474 remains an open question. This is particularly relevant when examining features of proteins
475 such as the genome maintenance protein LANA, which is expressed in all KSHV-infected
476 cells and malignancies, making it an attractive immunological target. Like its related genome
477 maintenance protein homologue in EBV, EBNA1, LANA encodes extensive repeat

478 sequences, some of which share nucleotide homology with EBNA1. These function to limit
479 protein synthesis *in cis* and inhibit proteasomal degradation (34, 41-43). As most CD8+ T
480 cell epitopes appear to be derived from newly synthesized proteins which are degraded by the
481 proteasome (44), these features of LANA have been shown to minimize CD8+ T cell
482 recognition of model epitopes inserted into LANA (33, 34, 41). Although LANA-specific
483 CD8+ T cell recognition of KSHV-infected cells is untested, one may predict recognition to
484 be minimal. By contrast, epitope generation for CD4+ T cell recognition would not be
485 subject to these restrictions and as B lymphocytes express class II MHC, these may be
486 legitimate targets for LANA-specific CD4+ T cells. Indeed we found that for all T cell
487 specificities tested, infected cells could be recognized by the MHC matched T cell clones.
488 The magnitude of responses are comparable to those seen in analogous experiments using
489 EBV-specific CD4+ T cells targeting epitopes from some latent antigens such as the EBNA3
490 proteins expressed by EBV transformed B lymphoblastoid cell lines (LCLs) (45-48).

491 The ability of the LANA-specific CD4+ T cell clones to recognize KSHV-infected cells
492 shows some contrast to what is seen using EBV EBNA1-specific CD4+ T cells challenged
493 with their cognate LCL which naturally expresses EBNA1. These effectors either do not or
494 weakly recognize such targets (47, 49). This is thought to be a consequence of the poor
495 release of EBNA1 protein by LCLs, thereby restricting the ability of cells to take up antigen
496 and re-present it to T cells, or making EBNA1 epitope presentation reliant on endogenous
497 processing of antigen through a macroautophagy route (48, 50, 51). We have previously
498 shown that B cells with intact antigen processing pathways exogenously fed LANA protein
499 can take up, process and present epitopes to LANA-specific CD4+ T cells and that such B
500 cells can also present endogenously expressed LANA (6). These findings suggest that
501 LANA, despite performing a similar function and having some sequence homology to
502 EBNA1 is processed and presented differently and may be a more relevant target for CD4 T

503 cells as compared to EBNA1 in EBV infection. Furthermore, we suggest that as LANA has
504 properties which limit efficient CD8+ T cell recognition, CD4+ T cell immunity to this
505 protein is likely to be important in control of KSHV infection and disease.

506

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660

661 Figure legends

662 Figure 1. Frequency of KSHV-infected B cells after ex vivo infection. Tonsillar B cells from
663 donors T46 and T7 were either infected with KSHV by culturing on monolayers of induced
664 VK219 cells for 48 hours, or mock infected by culturing on induced monolayers which had
665 been pre-treated with phosphonoacetic acid (PAA) to inhibit virus replication. B
666 lymphocytes were then magnetically sorted, cultured for a further 72 hours to allow
667 expression of the GFP reporter from the rKSHV.219 genome, then stained for CD20 surface
668 expression and the proportion of cells expressing GFP as a marker of infection determined by
669 flow cytometry.

670 Figure 2. Expression of LANA protein in B cell lines by immunofluorescent staining
671 analysis. Mock infected or KSHV-infected cells which had been established for eight weeks
672 were stained with a LANA-specific monoclonal antibody and bound antibody detected by
673 staining with an Alexa-fluor conjugated secondary antibody. Antibody binding was
674 visualized by epifluorescence microscopy.

675 Figure 3. Western blot analysis for detection of viral proteins in lysates from B cell lines.
676 Proteins from lysates of the PEL JSC-1, mock infected and KSHV-infected B cell lines which
677 had been maintained for 18 weeks were separated by PAGE and blotted onto PVDF

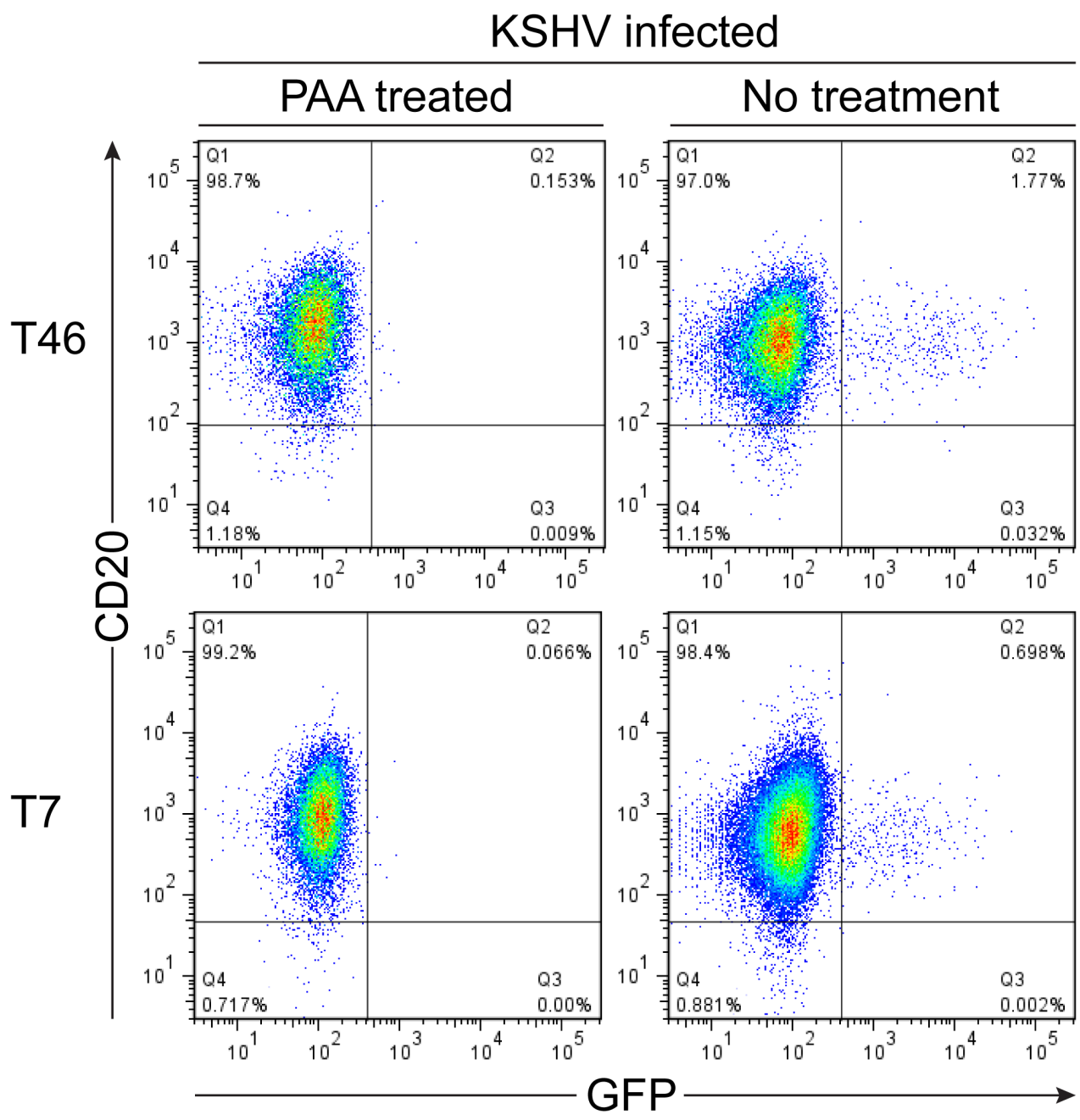
678 membranes. Blots were sequentially probed for either LANA, vIRF3 or actin. Filled
679 arrowheads represent specific protein detection.

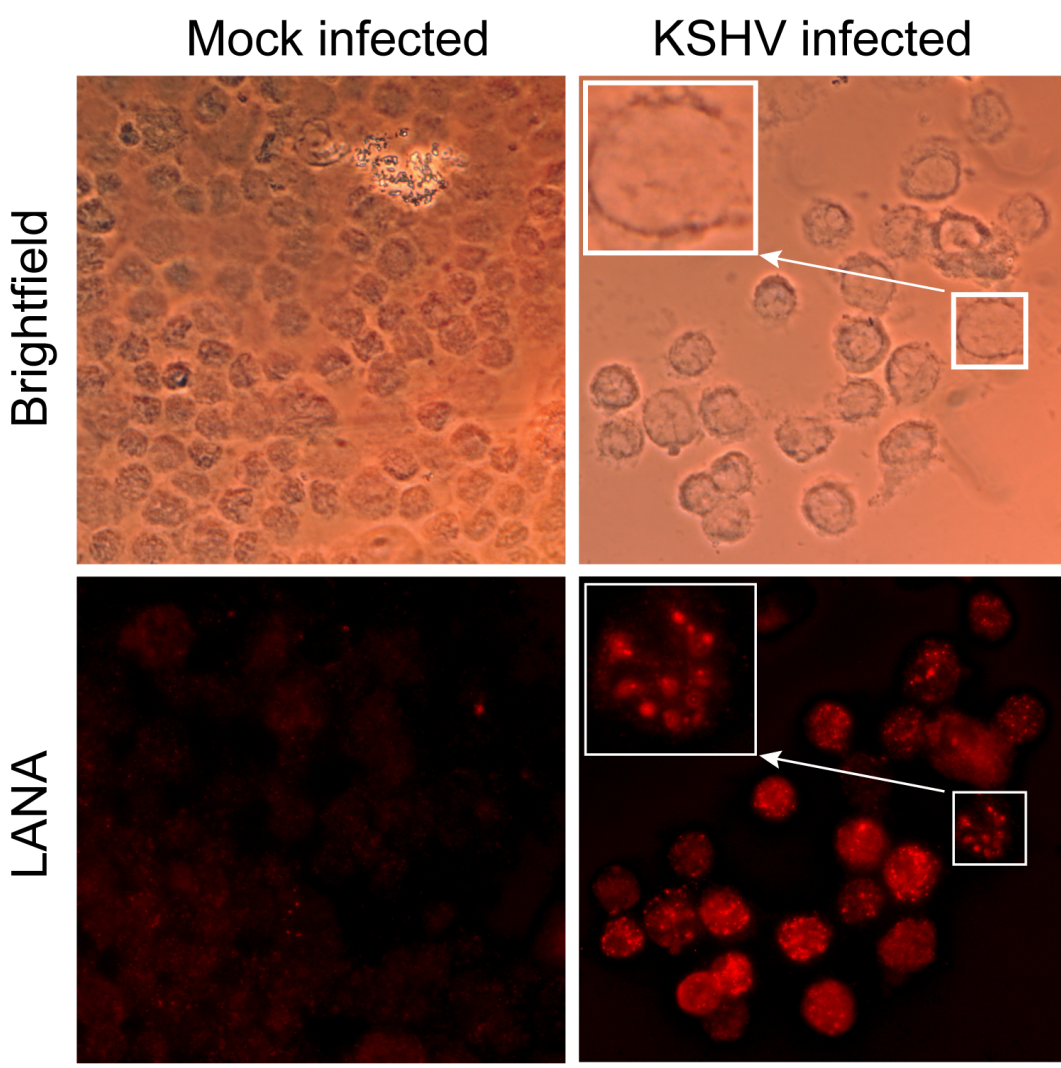
680 Figure 4. Gene expression analysis and genome load within KSHV infected cell lines. A.
681 Map of qPCR assays to detect splice variants driven from the constitutively active promoter
682 of the latency locus. Arrows represent primers and dashed lines indicate intronic regions
683 spanned by the primers. B. Gene expression was determined from cDNA prepared from the
684 PEL lines BC-1, JSC-1, BCBL-1, VG-1 and BC-3 and the infected B cell lines, assayed for
685 expression of the following transcripts: tricistronic LANA-vCyclin-vFLIP, bicistronic
686 vCyclin-vFLIP, monocistronic vFLIP, vIL-6, vIRF3, K3, K5, ORF50 and GAPDH.
687 Transcripts are expressed relative to GAPDH transcript abundance. * denotes $<10^{-5}$ vFLIP
688 transcript detected for T3 and T10. C. Genome load was determined in DNA extracted from
689 the above cell lines and quantified using the vIL-6 qPCR assay. Results shown for the
690 infected B cell lines have been corrected to represent 100% GFP expression. Infected
691 lymphocytes had been established for the following times: T3 18 weeks, T10 18 weeks, T13
692 10 weeks, T14 8 weeks, T32 10 weeks, T42 12 weeks, T44a 10 weeks, T44b 8 weeks, T48 6
693 weeks.

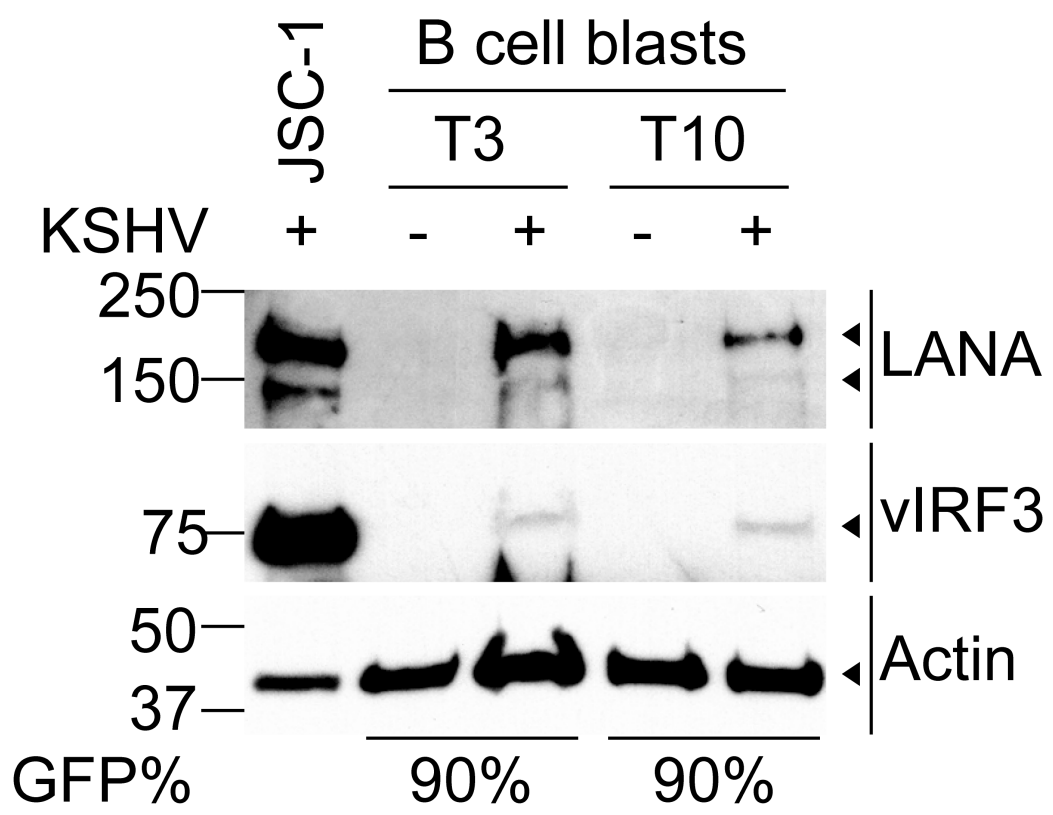
694 Figure 5. Cell surface immunoglobulin usage by KSHV-infected B lymphocytes. A.
695 Tonsillar cells were infected with KSHV as described in Figure 1 and stained for the
696 expression of CD20, Ig λ and IgM, followed by flow cytometry analysis. Populations shown
697 are gated on the CD20 GFP negative uninfected cells or CD20 GFP positive infected
698 population. B. B cell lines which were either mock- or KSHV-infected and maintained for
699 twenty weeks were analyzed for cell surface expression of Ig λ and IgM. Mock infected cells
700 are gated on viable cells, while KSHV infected cells are gated on GFP viable cells.

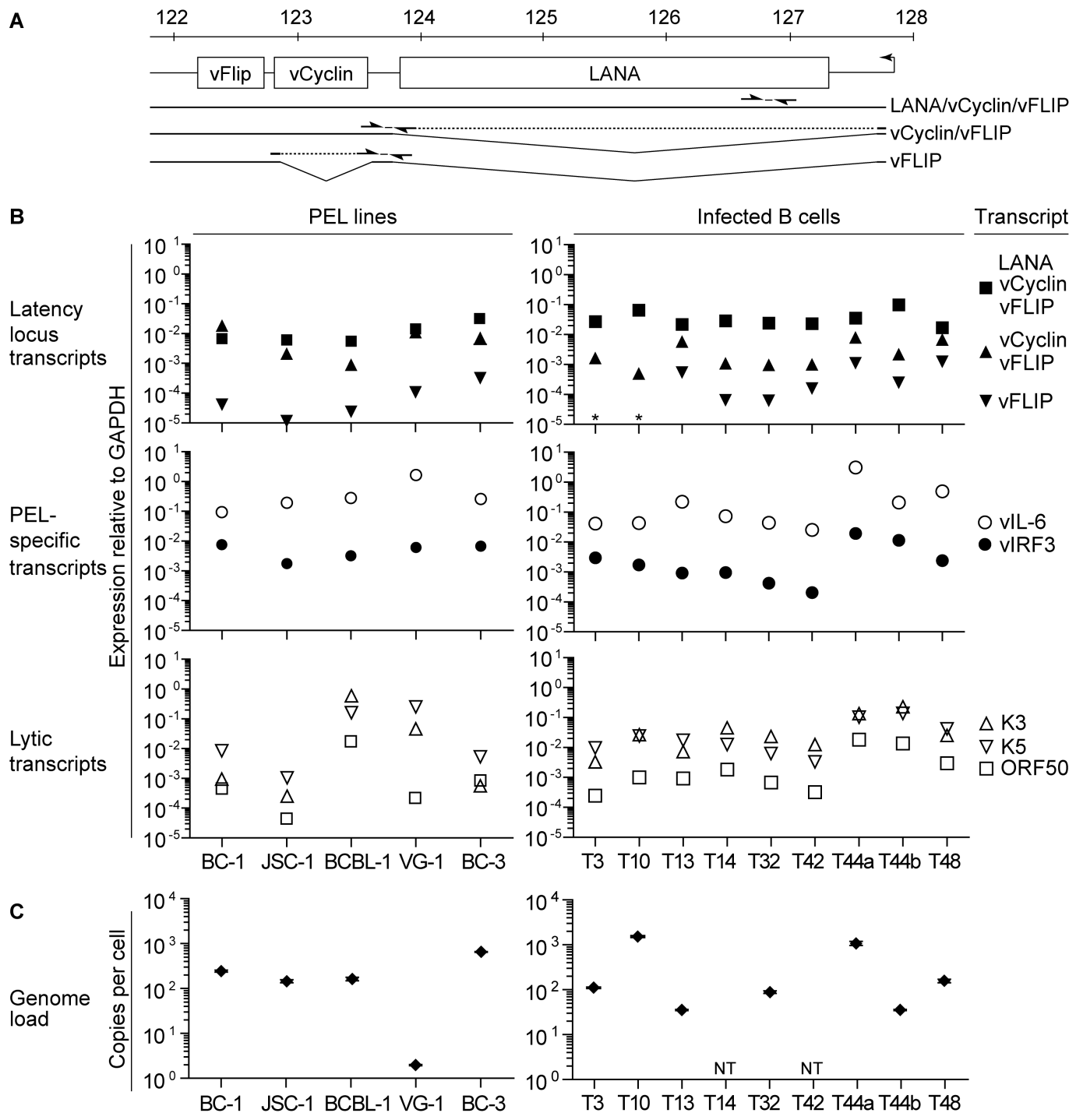
701 Figure 6. Flow cytometric analysis of cell surface expressed immunological markers on
702 KSHV-infected B cell lines. A. KSHV-infected B cell lines which had been established for
703 eight weeks were stained with antibodies specific to either MHC class I or class II or an
704 isotype control antibody (Iso) and gated on either GFP positive infected cells (KSHV) or GFP
705 negative cells co-resident in the culture (Non). B. KSHV-infected B cell lines were stained
706 for expression of CD86 or CD54 and gated on infected and co-resident non-infected cells as
707 before. C. KSHV-infected cell lines that had been established for six weeks were infected
708 with either a K5 deleted virus (K5Δ) or a virus derived from this construct in which the K5
709 gene was restored (K5R) and stained for expression of CD86 or CD54 on infected and co-
710 resident non-infected cells as before. Data is representative of at least three independent cell
711 lines for each assay.

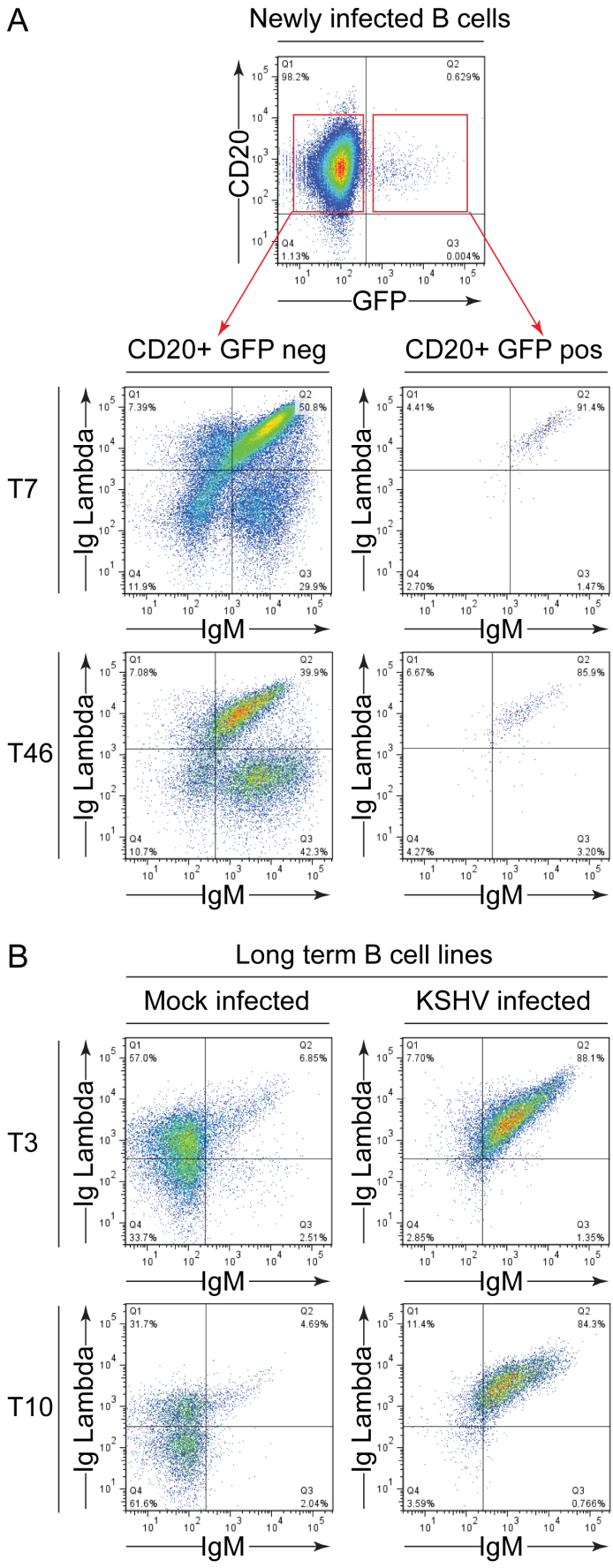
712 Figure 7. LANA-specific CD4+ T cell recognition of mock- or KSHV-infected B lymphocyte
713 lines. A. KSHV-infected B cell lines or mock infected B cell lines or mock infected B cell
714 lines sensitized with the T cells cognate peptide (Pep) derived from donors T7 and T31 which
715 co-expressed HLA-DQ6 and HLA-DR13 were challenged with the LANA-specific HLA-
716 DQ6 restricted clones LAP/LRS, EYR, PAF or the DR13 restricted clones LRS and GDD. B
717 lymphocyte lines had been established for ten weeks. B. KSHV-infected or mock infected B
718 cell lines derived from donor T10 which expressed HLA-DQ7 were challenged with the
719 LANA-specific HLA-DQ7 restricted clone GSP. B lymphocyte lines had been established for
720 eight weeks. In both cases T cell recognition of targets was assessed by measuring IFN- γ
721 secretion. Error bars represent the standard deviation of assay replicates.

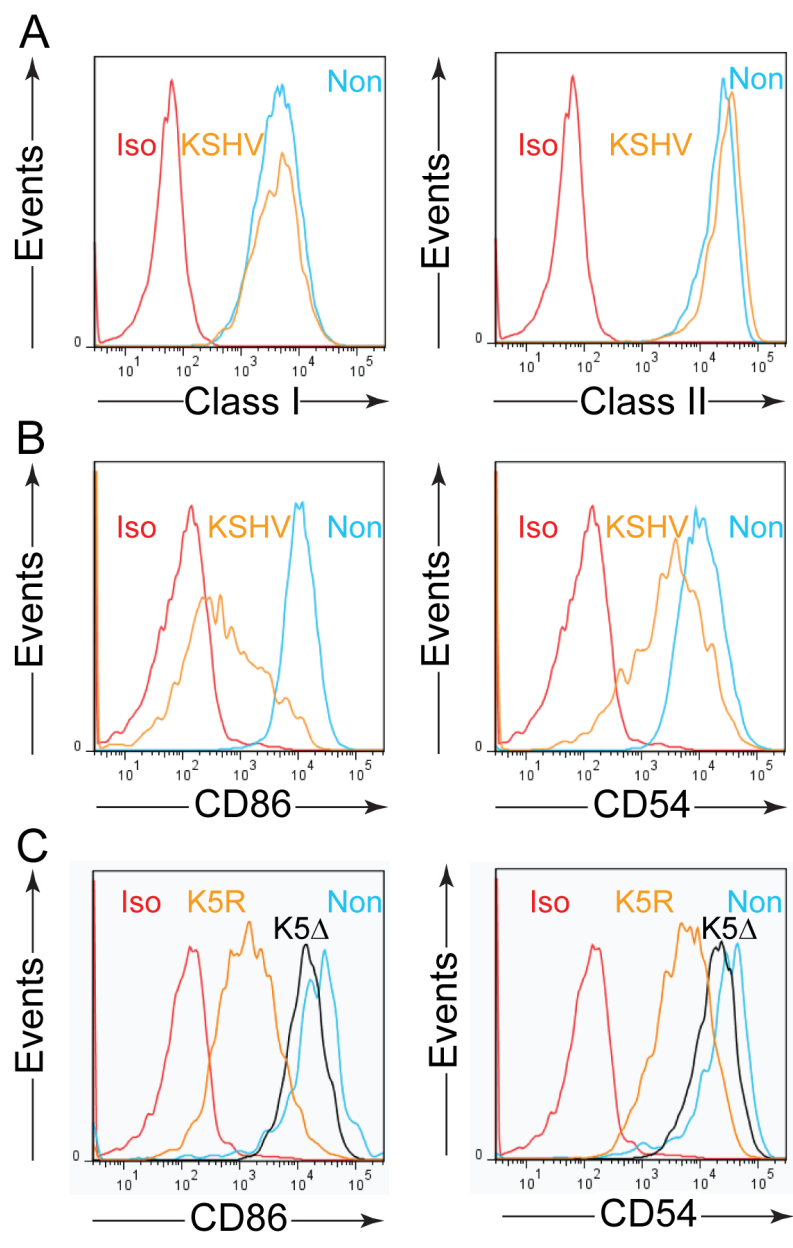












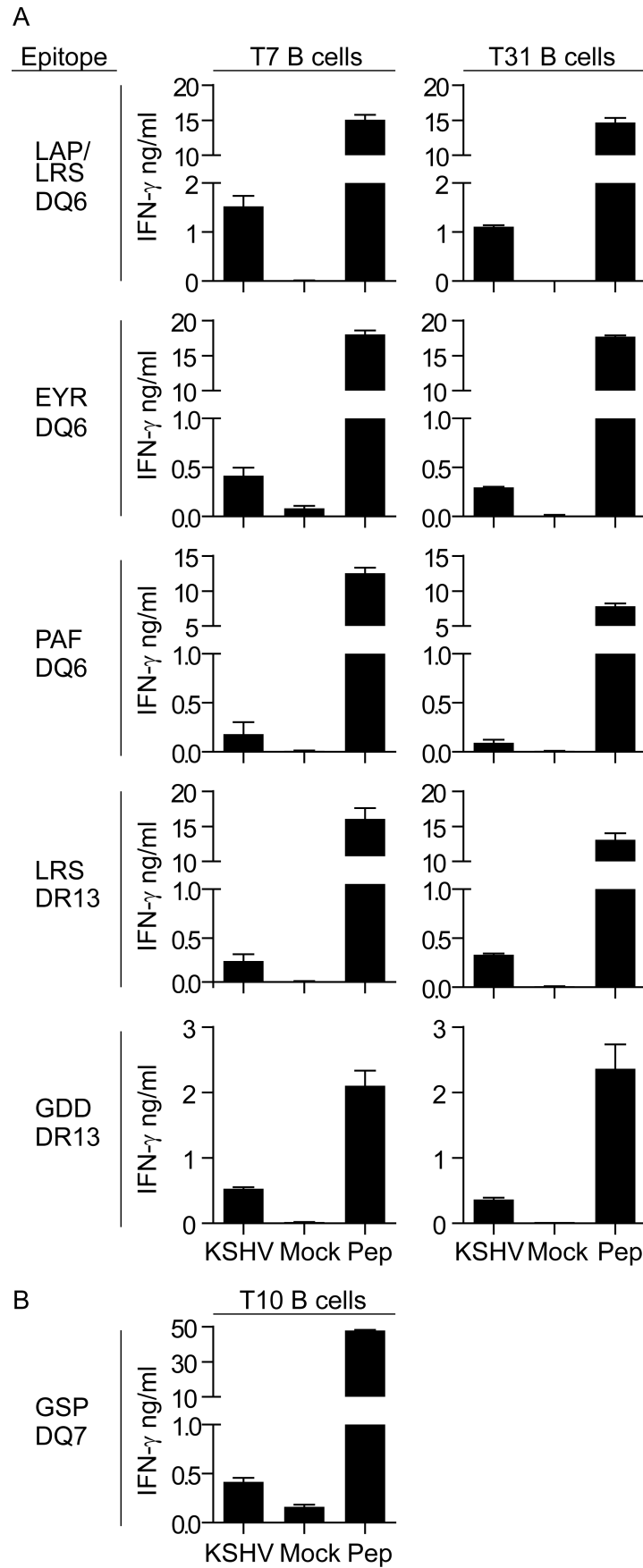


Table 1 qPCR assay primer and probe sequences with BC-1 genomic locations

Assay	Forward primer	Probe	Reverse primer
LANA/	TTTACCTCCACCGCACTCT	CACGTCTTCTCCCAATCCCTCC	GTCCCCGAGACACAGGAT
vCyclin/	127007-126988	126974-126951	126927-126945
vFLIP			
vCyclin/	GGTAGATGGGTCGTGAGAACT	ACCGTCGCGCTCCGCACTT	TCCGGCTGACTTATAAACA^AAGC
vFLIP	123692-123714	123771-123752	127831-127813^123776-123773
vFLIP	TTCCACTGCCGC^CTGTAGAG	TGTCAGGTTCTCCATCGACGACG	ACGGACAACGGCTAGCGTACT
	122848-122859^123595-123602	123623-123646	123679-123659
vIL-6	GATGCTATGGGTGATCGATGAA	TTCCGCGACCTCTGTTACCGTACCG	GGGCTCTAGAATACCCCTGCAGAT
	17753-17732	17728-17704	17678-17701
vIRF3	GGAGAAGACCA^AGGCCATTG	TGAGGAGGATCACCCAGCCTTTTGC	CTGCGTGACCGGCACAT
	90952-90942^90847-90838	90815-90791	90773-90789
ORF50	GCAAGATGACAAG^GGTAAGAAGC	CTGTGTGGAAAGCTTCGTCGGCCTC	TGGTAGAGTTGGGCCTTCAGTT
	71601-73613^72572-72581	72592-72616	72645-72624
K3	GCGGGTTGAAGTGTTCAT	TCGCGCGACATCACCAGAGTGTG	TTTCCTGAAGCTCGATCTCCTCTA
	19139-19120	19112-19090	19063-19086
K5	TCCACCCGCAGTGTTTAAGC	TGTCTCGAAACACGGCCTGTCAAATG	CGTGCGCGTGCGGTATA
	26368-26349	26335-26310	26283-26299

^ denotes splice junction.